

Supplemental Material to:

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**Epigenetic regulation of miR-21 in colorectal cancer:
ITGB4 as a novel miR-21 target and a three-gene network
(miR-21-ITGB4-PCDC4) as predictor of metastatic tumor
potential**

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article/26842/](http://www.landesbioscience.com/journals/epigenetics/article/26842/)**

Figure S1

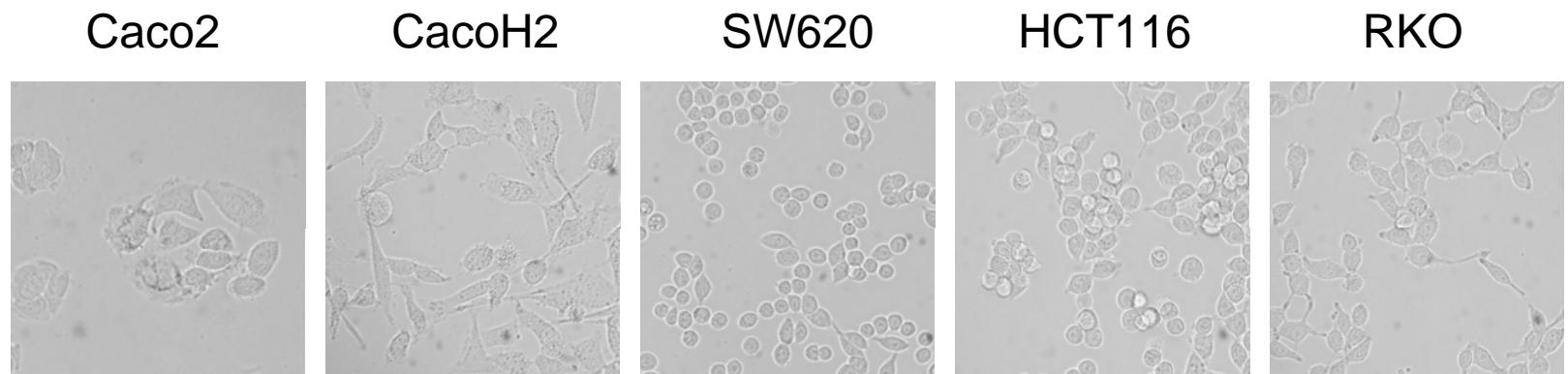


Figure S1. Phase contrast microscopy pictures of cell lines with EMT traits (Caco-H2, SW620, HCT116 and RKO) and with epithelial phenotype (Caco-2).

Figure S2

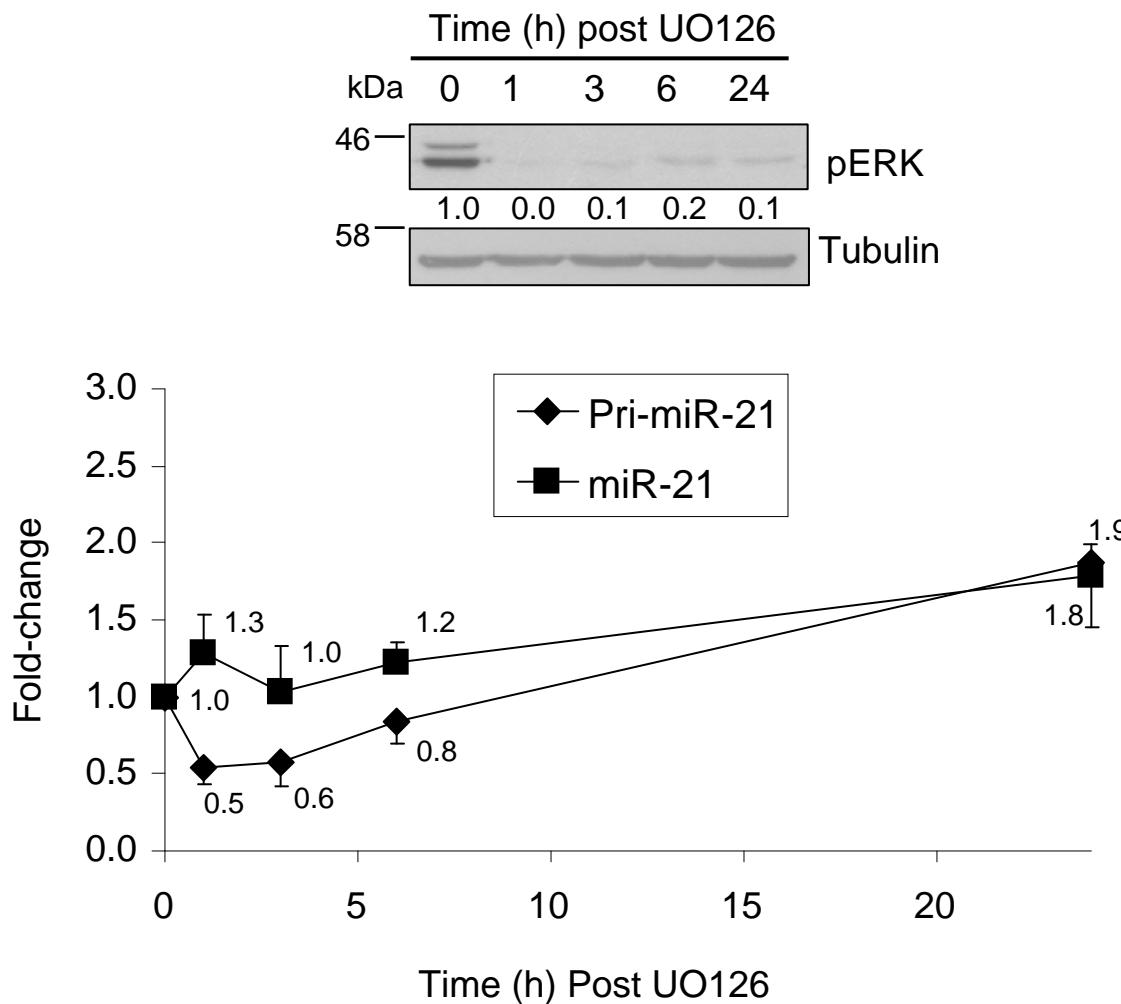


Figure S2. Top, Time course of ERK1/2 phosphorylation inhibition using UO126 at a final concentration of 40 μ M on HCT116 cell line. Bottom, Pri-miR-21 and mature miR-21 levels at different time point of UO126 treatment. Pri-miR-21 abundance was reduced by 50% 1 hour after treatment, maximum ERK1/2 inhibition.

Figure S3

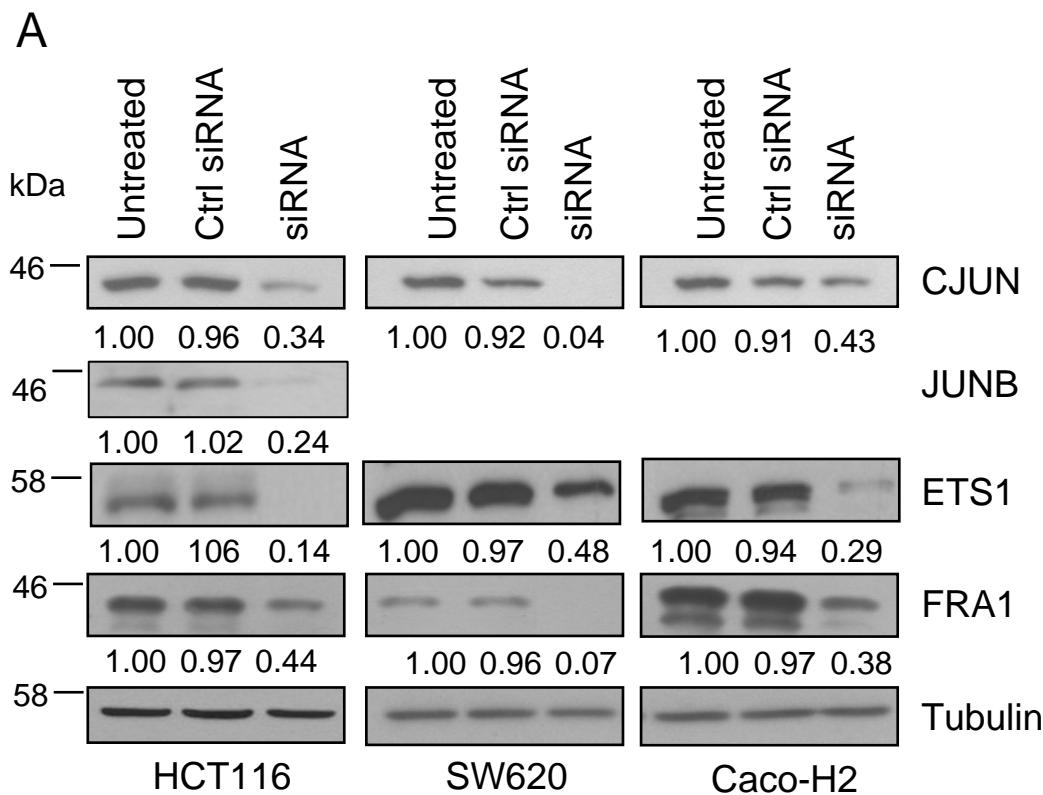


Figure S3. A. SiRNA of AP-1 and ETS1 transcription factors in EMT cell lines and subsequent WB analysis. Due to the low expression (almost undetectable, see Figure 2B) of JUNB in SW260 and CacoH2 siRNA assay for this factor was not performed.

Figure S3

B

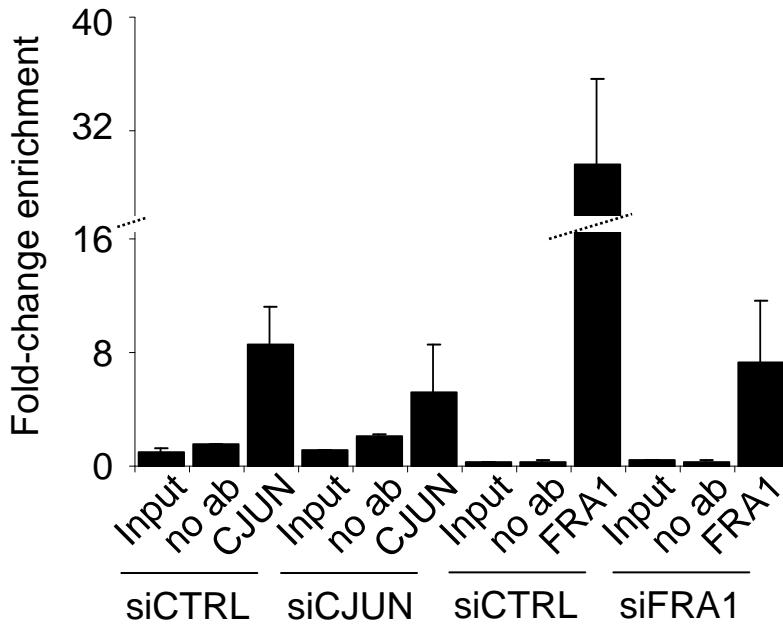


Figure S3. B. Occupancy of CJUN and FRA1 on miR-21 promoter gene after siCJUN and siFRA1 in HCT116. Both factors were found displaced form miR-21 after silencing.

Figure S4

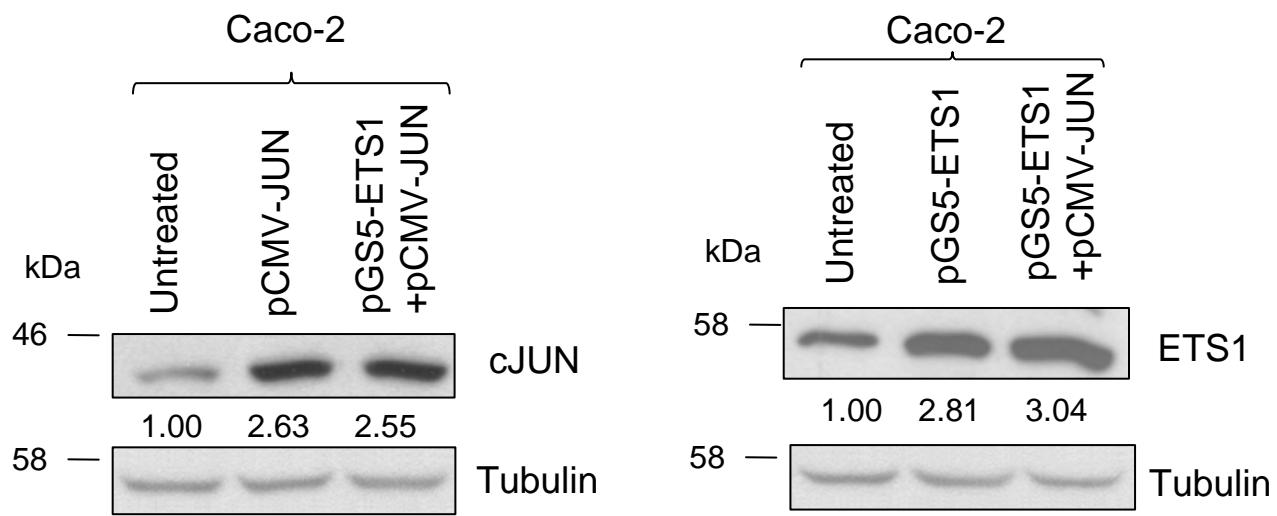
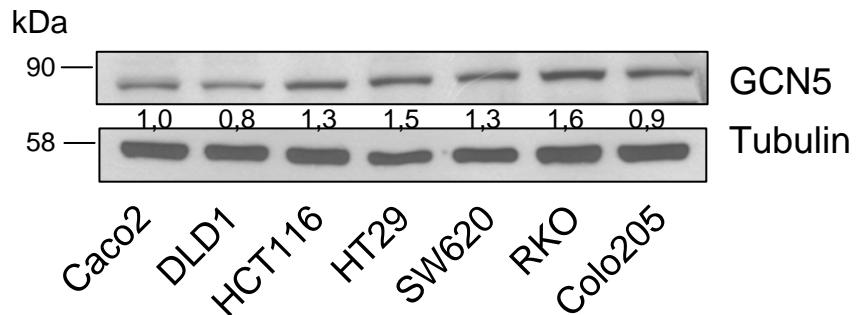


Figure S4. Transient over-expression of CJUN and ETS1 alone and in combination in Caco-2 cell lines.

Figure S5

A



B

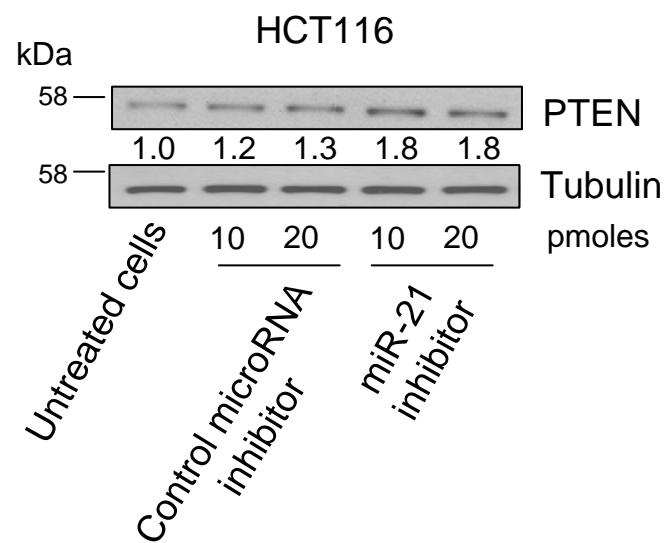


Figure S5. A. WB analysis of GCN5 protein levels in CRC cell lines. B. The performance of miR-21 inhibitor was tested by WB analysis on a known miR-21 target (PTEN) using different concentration (10 and 20 pmoles) on HCT116 cell lines.

C

Figure S5

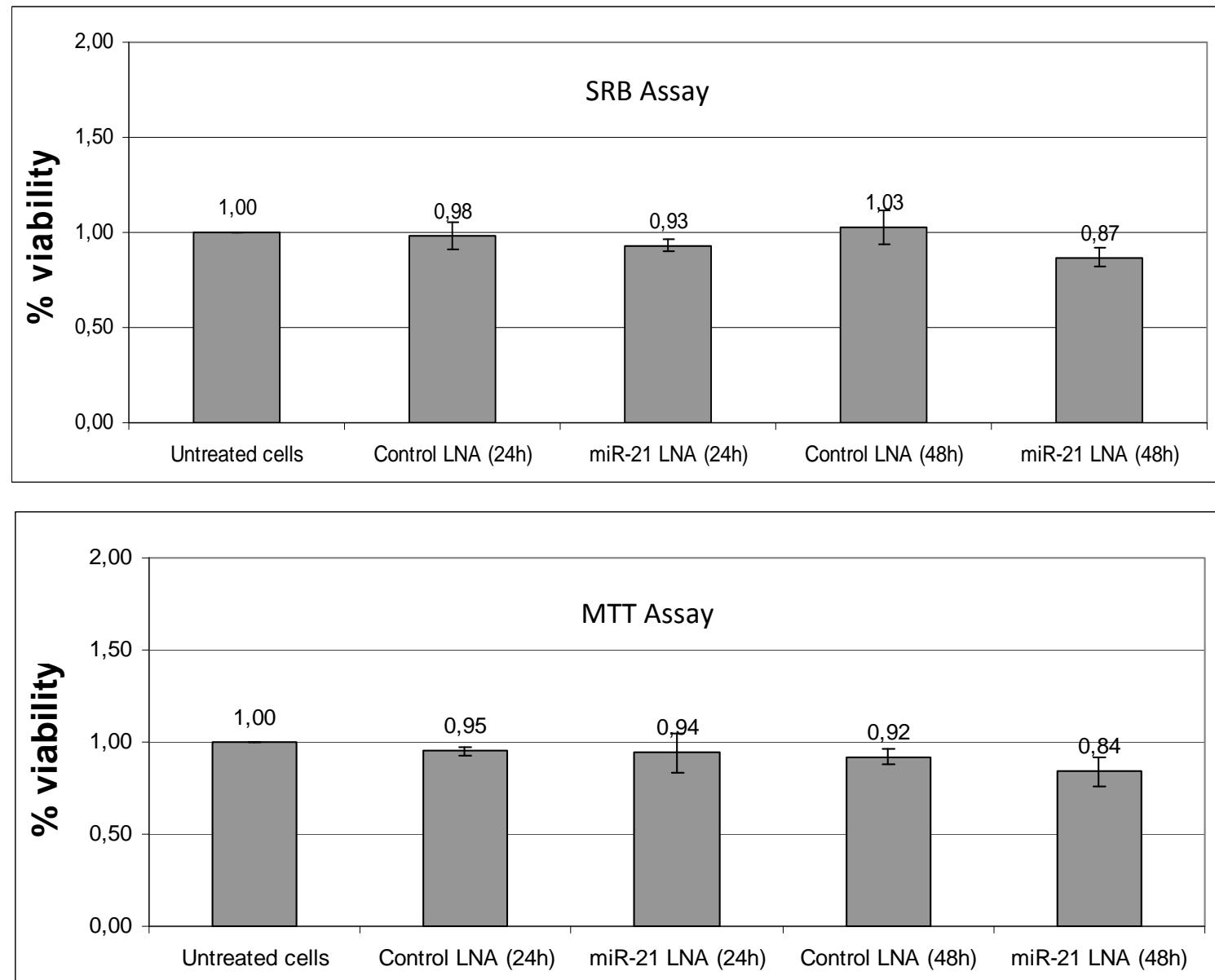


Figure S5. C. Viability of HCT116 cells after miR-21 inhibitor treatment was evaluated using SRB and MTT assays 24 and 48 hours post transfection.

Figure S6

A

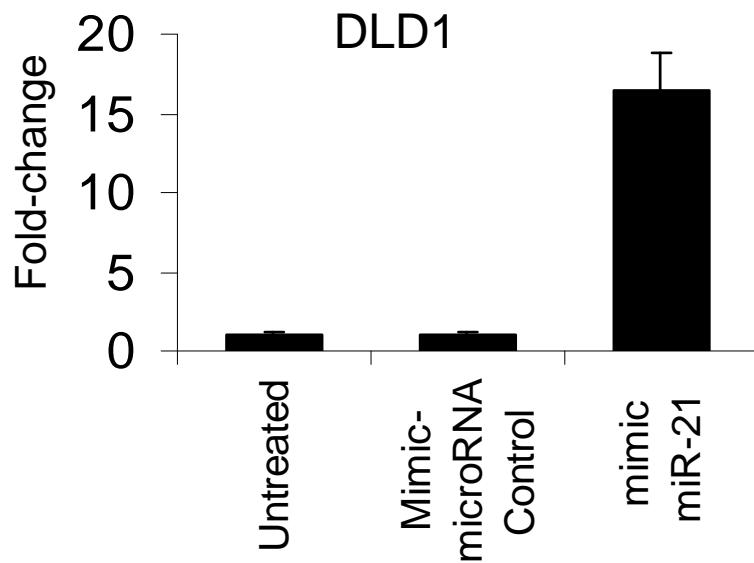


Figure S6. A. Mir-21 expression in DLD1 cell line after transient transfection with miRvana mimic control and mimic-miR-21, respectively. Mir-21 was found over expressed by 16,45 fold after 24 hours.

Figure S6

B

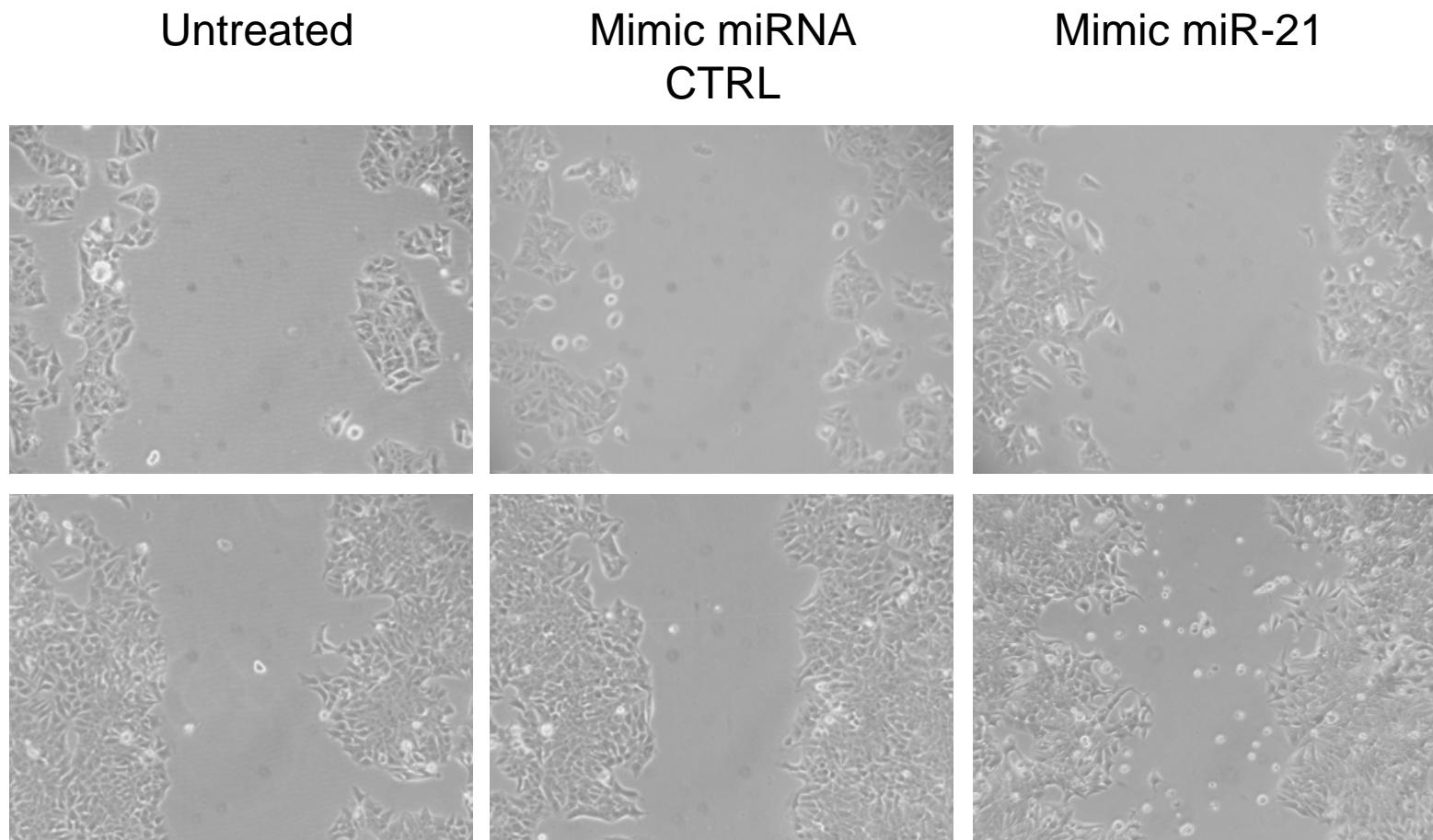


Figure S6. B. Wound healing assay performed on DLD1 cell line after transient over expression miR-21. MiR-21 over expression enhanced cell mobility and changed the cell-body shape of DLD1 cell line conferring them a mesenchymal phenotype.

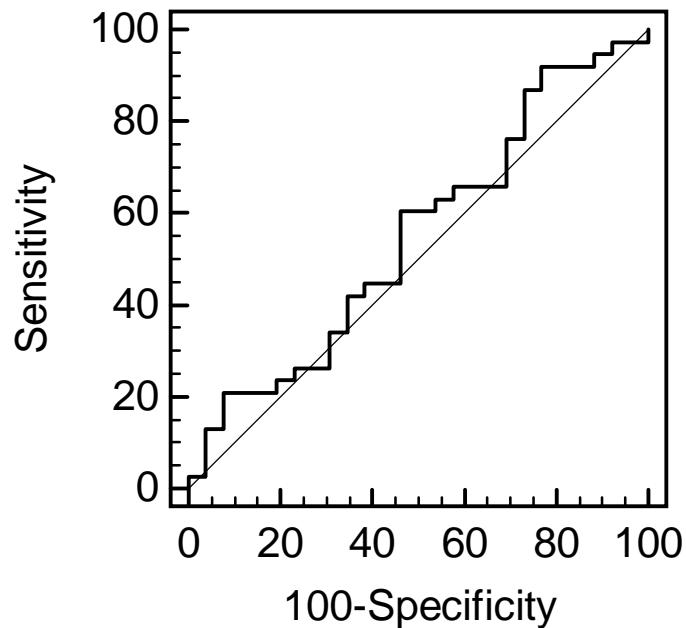
Figure S7

| mirNA ID | Location | Hybridization | Free energy | Score |
|-------------|------------------|--|-------------|--------|
| hsa-miR-21 | <u>735~757</u> | mirNA: 3' agUUGUAGU-CAGACUAUUCGAu 5' : Target:5' cgGACATGAGGCCTGAGAAGCTg 3' | -16.80 | 140.00 |
| | <u>4774~4795</u> | mirNA: 3' uguCGGGU-AGCUGACCACAAc 5' Target:5' cacGCCAACCCGCCTGGTGTTC 3' | -22.40 | 166.00 |
| hsa-miR-21* | <u>5112~5134</u> | mirNA: 3' ugucGGGU--AGCUGACCACAAc 5' : Target:5' gtgcCCCAGGCCCCGCTGGTGTTC 3' | -18.40 | 155.00 |
| | <u>4749~4769</u> | mirNA: 3' uguCGGGUAGCUGACCACAAc 5' : : Target:5' ctcGCCTGACTGCTGGTGTgc 3' | -16.80 | 142.00 |

Figure S7. Hybridization sites of miR-21 and miR-21* on ITG β 4 mRNA coding sequence.

Figure S8

A



| Gene | AUC | SE | P (Area=0.5) |
|-----------------------------------|--------|---------|--------------|
| <i>3-gene logistic regression</i> | 0,5506 | 0,07495 | 0,4995 |

Figure S8. A. ROC curve analysis of undivided samples performed combining the quantitative PCR data sets for miR-21, ITGB4 and PDCD4. Area under the curve (AUC), standard error (SE) and p-value are showed in the enclosed table.

Figure S8

B

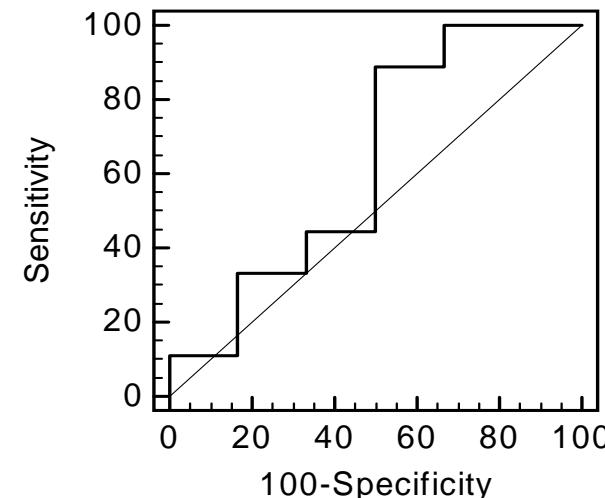
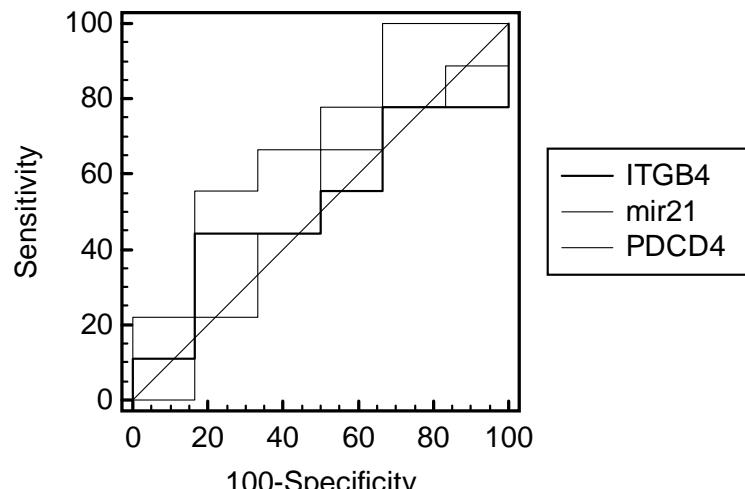


Figure S8. B. ROC curves analysis carried out on quantitative PCR data sets (left single-gene assays, right after 3-gene combination) for those samples presenting normal *ITGB4* expression.

Figure S8

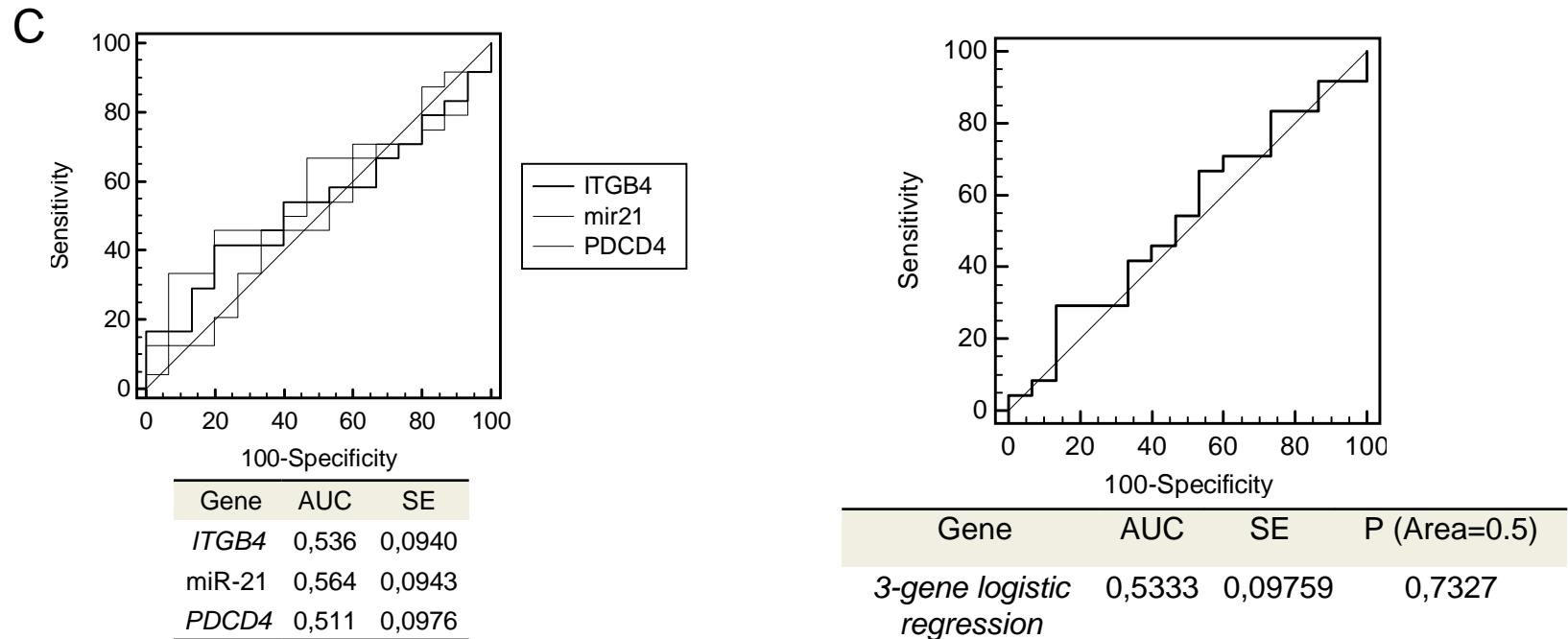


Figure S8. C. ROC curves analysis carried out on quantitative PCR data sets (left single-gene assays, right after 3-gene combination) for those samples presenting *ITGB4* up-regulation. Area under the curve (AUC), standard error (SE) and p-value are shown in the enclosed tables.

Table S1. Summary of EMT and metastatic properties of CRC cell lines^(39, 56). N/A= not assigned

| | Caco2 | CacoH2 | SW620 | HCT116 | RKO |
|--------------------------|---------------------------------|--|---|----------------------|----------------------|
| Tumor origin | Colon | Caco-2 stably overexpression <i>HRAS</i> mut. oncogene | Colon; Derived From Metastatic Site: Lymph Node | Colon | Colon |
| Stage | Colorectal Intermediate Adenoma | N/A | Dukes' Type C, Colorectal Adenocarcinoma | Colorectal carcinoma | Colorectal carcinoma |
| Migration Transwell test | None | High | Median | High | High |
| E-cadherin expression | High | Absent | Very low | Very low | Absent |
| Vimentin expression | Absent | High | High | Low | Low |
| ITGB4 expression | Moderate | Low | Low | Low | Low |

Table S2. List of primers used for mRNA expression analysis and ChIP-coupled quantitative PCR analysis.

| Name | Primer Forward | Primer reverse |
|-----------------|----------------------|-------------------------|
| GAPDH | GAAGGTGAAGGTCGGAGT | CATTGGGTGGAATCATATTGGAA |
| ITGB4 | TTCAATGTCGTCTCCTCCAC | CAATAGGTGGTTGTCATCG |
| Pri-mir-21 | ATCAAATCCTGCCTGACTG | AAAGACTCTAAGTGCCACC |
| GCN5 | CTATGGGGCAAACCTCTCAA | ATCCTCCAGGGTCAGGTTCT |
| miR-21 promoter | TTAACTAGGGATGACAAAGC | AAGTCCCACATTATCACAC |

Table S3. List of antibody used for WB and ChIP analysis.

Tubulin (TU-02) (sc-8035, Santa Cruz Biotechnology Inc.)
Phospho-Akt (Ser473) (#9271, Cell Signaling Technology Inc.)
Phospho-MEK1/2 (Ser217/221) (#9121, Cell Signaling Technology Inc.)
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) (#9106, Cell Signaling Technology Inc.)
CJUN (H-79) (sc-1694, Santa Cruz Biotechnology Inc.)
JUNB (C-11) (sc-8051, Santa Cruz Biotechnology Inc.)
JUND (329) (sc-74, Santa Cruz Biotechnology Inc.)
Ets-1 (C-20) (sc-350, Santa Cruz Biotechnology Inc)
Fra-1 (R-20) sc-605, Santa Cruz Biotechnology Inc.)
Fra-2 (L-15) (sc-171, Santa Cruz Biotechnology Inc.)
E-cadherin (H-108) (sc-7870, Santa Cruz Biotechnology Inc.)
GCN5 (H-75) (sc-20698, Santa Cruz Biotechnology Inc.)
ITGB4 (A9) for WB and Immunoassay (sc-13543, Santa Cruz Biotechnology Inc.)
ITGB4 for blocking assay (MAB2058, Chemicon-Millipore)
H3K27m3 (07-449, Millipore)
H3K27ac (ab4729, Abcam)
H3K9-14ac (06-599, Upstate)
H3K9me2 (07-441, Millipore)
H3K4me3 (ab8580, Abcam)